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Two Simple Methods for the Collection of Individual Life Stages of Reniform Nematode, *Rotylenchulus reniformis*

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Abstract: The sedentary semi-endoparasitic nematode *Rotylenchulus reniformis*, the reniform nematode, is a serious pest of cotton and soybean in the United States. In recent years, interest in the molecular biology of the interaction between *R. reniformis* and its plant hosts has increased; however, the unusual life cycle of *R. reniformis* presents a unique set of challenges to researchers who wish to study the developmental expression of a particular nematode gene or evaluate life stage–specific effects of a specific treatment such as RNA-interference or a potential nematicide. In this report, we describe a simple method to collect *R. reniformis* juvenile and vermiform adult life stages under in vitro conditions and a second method to collect viable parasitic sedentary females from host plant roots. *Rotylenchulus reniformis* eggs were hatched over a Baermann funnel and the resultant second-stage juveniles incubated in petri plates containing sterile water at 30°C. Nematode development was monitored through the appearance of fourth-stage juveniles and specific time-points at which each developmental stage predominated were determined. Viable parasitic sedentary females were collected from infected roots using a second method that combined blending, sieving, and sucrose flotation. *Rotylenchulus reniformis* life stages collected with these methods can be used for nucleic acid or protein extraction or other experimental purposes that rely on life stage–specific data.

Key words: host-parasitic relationship, life stages, reniform nematode, *Rotylenchulus reniformis*, technique.

The reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) is a sedentary semi-endoparasitic pathogen of more than 300 plant species grown in the tropical, subtropical, and warm-temperate regions of the world (Robinson et al., 1997). *R. reniformis* is a serious pest of a number of crop species including Upland cotton, soybean, pineapple, and sweet potato (Gaur and Perry, 1991). During the 2011 growing season, cotton producers in the United States lost approximately 279,000 bales of cotton to *R. reniformis* infection having an estimated value of more than $90 million (Blasingame and Patel, 2012). Reasons for such huge losses include the lack of resistant varieties and the ability of *R. reniformis* to survive under adverse environmental conditions in the absence of a host (Robinson et al., 2005).

Unlike the sedentary endoparasitic nematode genera, i.e., *Heterodera*, *Globodera*, and *Meloidogyne*, infection by *R. reniformis* is not initiated by the second-stage juvenile (J2) but is instead accomplished by the vermiform adult female; however, as with these other plant-parasitic nematodes, *R. reniformis* completes its first molt inside the egg and emerges as a J2. *Rotylenchulus reniformis* J2 display vigorous movement and show a well-formed distinct stylet; however, this period of motility is short-lived with the J2 eventually becoming less active and assuming a crescent shape (Nakasono, 1973; Bird, 1984). During the molt of the J2 to the third-stage juvenile (J3), following the onset of nonmotility, the J2 stylet disappears and the J3 cuticle separates from the outer residual J2 cuticle (Nakasono, 1973; Bird, 1984). The most clearly identifiable morphological features of *R. reniformis* J3 are the presence of the residual J2 cuticle, lack of movement, and disappearance of the stylet. Further morphological changes take place during the molt from J3 to fourth-stage juvenile (J4). The primary identification feature of the *R. reniformis* J4 is the presence of the residual J2 and J3 cuticles (Nakasono, 1973; Bird, 1984). The emergence of *R. reniformis* male and infective female nematodes from the J4 is marked by the reappearance of a functional stylet and the anatomy of the esophageal region becoming distinct, particularly in the female (Bird, 1984). Male reniform nematodes do not feed, and they develop clearly discernible spicules. The vermiform *R. reniformis* female infects the host root tissue and starts feeding via the formation of a specialized feeding structure called a syncytium that is formed by the dissolution of cell walls of cells adjacent to the initial feeding cell (Heald, 1975; Rebois et al., 1975). Upon syncytium formation, the infective vermiform adult female becomes sedentary and eventually attains the characteristic reniform (kidney) shape. After fertilization by the male, the female deposits an average of 60 eggs within a protective gelatinous matrix that is secreted by the vaginal glands (Robinson et al., 1997).

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With the current lack of resistant cotton cultivars, control of *R. reniformis* field populations relies heavily on the use of various nematicides. Chemical compounds such as abamectin, thiodicarb, 1,3-dichloropropene, and oxamyl (Kinloch and Rich, 2001; Lawrence and McLean, 2002; Faske and Starr, 2006) along with biocontrol agents such as *Pasteuria* (Schmidt et al., 2010) have been tested and are being used in the management of *R. reniformis* and other plant-parasitic nematodes. Until recently, aldicarb was the primary *R. reniformis* control nematicide employed by producers; however, with its removal from the marketplace, alternative chemical control measures will need to be developed.

As an alternative to nematicide application, RNA-interference (RNAi) has shown promise as a biotech-based nematode control strategy. Many laboratories have reported significantly decreased plant-parasitic nematode reproduction following the RNAi-mediated silencing of esophageal gland-specific parasitism genes (Patel et al., 2008; Sindhu et al., 2009) or other genes necessary for nematode growth and development (Bakhetia et al., 2005; Klink et al., 2009). Regardless of the target gene, the success of RNAi hinges on determining at what stage in the nematode life cycle the target gene is expressed.

Established methods for the extraction of *R. reniformis* eggs from infected roots (Walters and Barker, 1993; Stetina et al., 1997) and for the isolation of mixed vermiform life stages from infested soil (Lawrence et al., 2005) are readily available and routinely used by nematologists. Despite the abundance of literature describing the *R. reniformis* lifecycle and juvenile development, an explicit method that outlines a means for isolating individual life stages in sufficient quantities for downstream molecular genetic or chemical assays has been lacking. In this study, we describe a simple method that allows the isolation of specific *R. reniformis* juvenile, infective, and parasitic life stages that can be used for any variety of downstream applications.

Materials and Methods

Reniform nematode cultures: Reniform nematode cultures were maintained on a susceptible cotton cultivar in a growth chamber. Two different culture conditions were followed for the collection of egg, sedentary female, and mixed vermiform life stages. To establish cultures for *R. reniformis* egg and sedentary female collection, germinated cotton seeds were sown in 10-cm-diam. clay pots containing autoclaved sand and vermiculite mixed in a 3:1 (sand:vermiculite) ratio. For the collection of mixed vermiform life stages, a heavier soil mix containing a 3:1 ratio of sand:silty loam soil with no vermiculite was used. Cotton seedlings were inoculated 2 wk after germination with a population of approximately 4,000 vermiform *R. reniformis* of mixed juvenile and adult life stages. Fourteen day-light hours with a temperature range of 28°C to 30°C were set in the growth chamber to facilitate optimal growth and transpiration. An average relative humidity of approximately 50% was maintained throughout the growth period of the plants. Miracle Gro (Scotts Company LLC, Marysville, OH) fertilizer (24-8-16) was applied twice a month as a source of nutrients at the rate of 3.9-ml fertilizer per liter water. Plants destined for egg and sedentary female collection were watered just enough to wet the top layers of sand and keep the leaves from wilting. Plants set aside for mixed vermiform nematode collection were watered until saturation. Pots were harvested to collect eggs and mixed vermiform life stages 8 wk after inoculation.

To collect mixed vermiform life stages of *R. reniformis* as a source of inoculum, the soil along with the roots of the host plant were carefully removed and washed in water to separate the root system from the soil. The vermiform *R. reniformis* were extracted from the soil by gravity sieving. Approximately three parts water per one part soil was mixed for 30 sec and left standing for another 30 sec to allow the larger soil particles to settle to the bottom while the nematodes along with lighter soil particles remained suspended in the water. The water-nematode suspension was decanted through an assembly of nested U.S. standard sieves (75-μm-pore sieve/44-μm-pore sieve). Vermiform nematodes collected on the 44-μm-pore sieve were used for inoculating culture pots as described above.

Egg collection and surface sterilization: Infected cotton roots were cut into 2- to 3-cm pieces and agitated in 1% sodium hypochlorite solution for 3 min as described by Hussey and Barker (1973). The solution containing the eggs was decanted over nested 75- and 25-μm-pore sieves and rinsed thoroughly with water. The eggs along with fine debris were washed off of the 25-μm-pore sieve into a beaker and further purified by sucrose flotation as described by Jenkins (1964) with slight modification. Eggs and contaminating debris were suspended in 40 mL of 35% sucrose by combining 20 ml of the egg-soil mixture with 20 ml of 70% sucrose in a 50-ml centrifuge tube. Immediately after mixing, 10 ml of water was layered over the sucrose-egg-debris solution and the tube was centrifuged at 1,260 × *g* for 10 min. The eggs were collected from the sucrose-water interface using a Pasteur pipette. Eggs collected from the interface were rinsed thoroughly with water and surface-sterilized in a 50-ml tube by washing in 0.01% $HgCl_2$ (Sigma-Aldrich, St. Louis, MO) for 5 min followed by a 45-min wash in 0.001% hibitane (chlorhexidine diacetate hydrate) (Sigma-Aldrich, St. Louis, MO). The surface-sterilized eggs were then washed three times with sterilized water to remove residual hibitane. Freshly hatched J2 were surface-sterilized in a similar fashion.

Collection of juvenile and vermiform adult life stages: To initiate the collection of *R. reniformis* vermiform life stages, approximately 100,000 eggs were layered over a single Kleenex® (Neenah, WI) tissue within a Baermann

funnel and incubated at 30°C. We had determined empirically that this number of eggs yielded a more consistent level of hatch under our experimental conditions (data not shown). J2 (Fig. 1A) were harvested from hatched eggs at regular 12-hr intervals. The J2 were surface sterilized to minimize microbial contamination during incubation for development into advanced stages.

Rotylenchulus reniformis juvenile development from J2 through adult was monitored in petri dishes on four separate occasions to determine the time-points of each stage of development under our experimental conditions. Aliquots containing approximately 20,000 surface sterilized J2 were placed in 9-cm-diam. petri plates and incubated in sterilized water at 30°C. Prior to addition of the J2, the petri plates were rinsed with 0.5% Tween-20 (Sigma-Aldrich, St. Louis, MO) solution to prevent the nematodes from adhering to the plate bottom. Nematode development was monitored for a period of 30 d to determine the optimum time-points for recovering each vermiform life stage.

Collection of sedentary parasitic females: Rotylenchulus reniformis sedentary parasitic females were collected from infected roots by first washing the roots free of soil with water. Approximately 15 g (fresh wt.) of clean roots were cut into 2- to 3-cm-long pieces and agitated with water in a household blender under the "blend" setting for four to five short pulses of 2 sec each. The blended mixture of roots and sedentary females was decanted over a nested assembly of four sieves (250-μm-pore/180-μm-pore/106-μm-pore/75-μm-pore) and washed thoroughly with water. Sedentary females free of egg masses (Fig. 1F) were collected primarily on the 106-μm-pore sieve with very few collected on the 75-μm-pore sieve along with some root debris. Egg masses collected on the 180-μm-pore sieve could be recovered for egg isolation. To further purify the sedentary females collected on the 106-μm-pore sieve and 75-μm-pore sieve from contaminating root debris, the sedentary female/root debris mixture was suspended in a 45% sucrose solution in a 50-ml tube, mixed by inversion, and centrifuged at 450 × *g* for 5 min. At this sucrose concentration the majority of sedentary females will remain suspended in the sucrose solution while heavier root debris will form a pellet. Following centrifugation, the supernatant was decanted over a 75-μm-pore sieve and washed thoroughly with water. The viability of the sedentary female nematodes following sucrose flotation was verified by observing metacorpal pumping under a stereomicroscope.

Results and Discussion

Using the methods described here, vermiform life stages of *R. reniformis* can be collected such that > 85% of specimens within a given sample represent a particular life stage. We observed that after 3 d of incubation at 30°C, the total percentage hatch of reniform nematode eggs was 70% to 75%. After 3 d, we found the hatch rate decreased considerably with the remaining minor fraction of fertile eggs hatching out by the

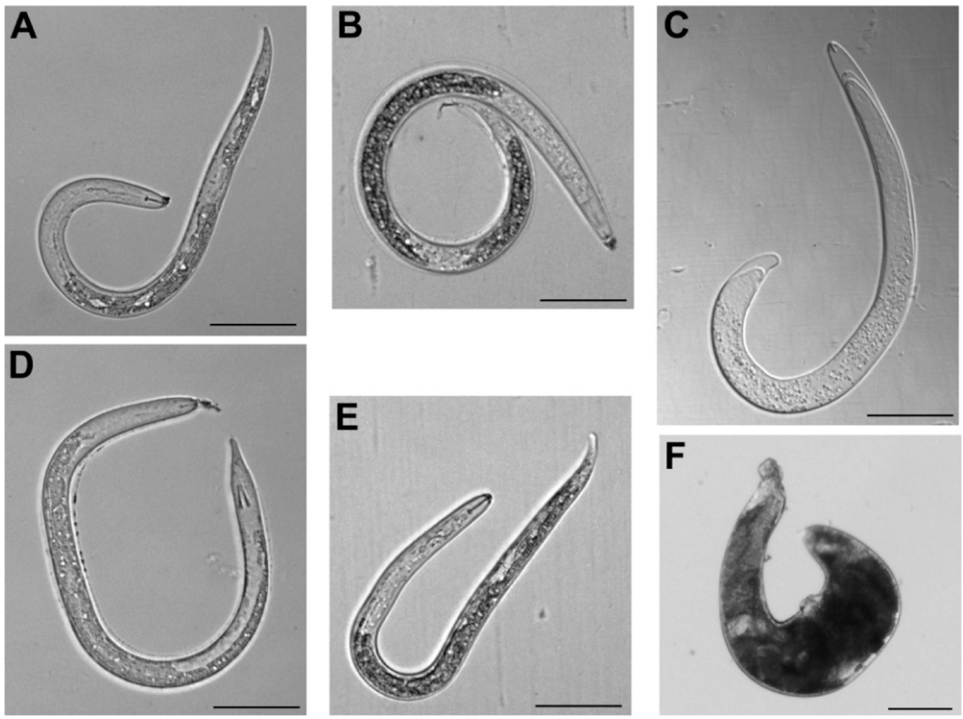

Fig. 1. Juvenile, vermiform adult, and parasitic life stages of *Rotylenchulus reniformis*, the reniform nematode: (A) second-stage juvenile, (B) third-stage juvenile, (C) fourth-stage juvenile, (D) vermiform adult male, (E) vermiform infective female, and (F) sedentary parasitic female. Scale bars for panels A to E equal 50 μm. Scale bar in panel F equals 100 μm.

end of day 6 or day 7. This hatching timeframe is similar to that previously observed for *R. reniformis* (Sivakumar and Palanisamy, 1976). Once hatched, we observed the progression of the J2 through the juvenile stages was relatively uniform with more than 85% of specimens within a sample corresponding to a specific life stage. Under our experimental conditions, samples comprised predominantly of J3 nematodes were readily apparent after 10 to 11 d of incubation of freshly hatched J2 in sterile water at 30°C (Fig. 1B). An additional 8 to 10 d of incubation were required for the appearance of the J4 life stage (Fig. 1C). Finally, vermiform adult male and female nematodes became evident after an additional 5 to 6 d of incubation following the appearance of the J4, i.e., total incubation time of 23 to 27 d (Fig. 1D and 1E). Previous studies of *R. reniformis* juvenile development had observed a time period of 14 to18 d for the appearance of vermiform males and females from J2 (Nakasono, 1973; Bird, 1984). While our data show a relatively slower course of juvenile development, it is important to note three ways in which our experiments differ from these earlier studies: (i) we charted nematode development on a population level and not on individuals, i.e., the timepoint at which a minimum of 85% of the specimens were of a specific life stage; (ii) we implemented a 30°C incubation temperature, while previous studies used 24–26°C; and (iii) we subjected J2 samples to a surface-sterilization protocol to minimize microbial contamination. Previous studies did not surface-sterilize their samples because the nematodes were observed on an individual basis (Nakasono, 1973; Bird, 1984).

For the characterization of parasitism-related genes, the collection of living sedentary parasitic *R. reniformis* females, free of contaminating soil and root debris, is absolutely required. Our method described here accomplishes this task by root blending, sieving, and sucrose flotation. From three independent extractions, starting from an average of 16.7 g of fresh root tissue, we calculated an average recovery of 372 sedentary females/g root. While the majority (approximately 75%) of sedentary females recovered were not viable for downstream applications because of damage sustained during the extraction process, the sheer volume of nematodes recovered by this method provides a more than adequate population of sedentary females from which viable specimens can be collected, e.g., 200 to 400 viable females for RNA extraction. While a method to collect root-associated *R. reniformis* has been reported (Stetina et al., 1997), this earlier method involved pouring the ground root mixture over nested 75- and 25-µm-pore sieves and collecting only the material on the 25-µm-pore sieve, while we found that the majority of parasitic female nematodes would not pass through a 106-µm-pore sieve. This observation may explain the low level of root-associated *R. reniformis* recovery experienced by these authors (Stetina et al., 1997).

The ability to collect specific *R. reniformis* vermiform life stages would aid in studying the expression pattern of genes responsible for the process of sexual differentiation. With the vermiform adult female being the infective life stage, the collection methodology described here would as well help in the characterization of putative parasitism genes expressed in the vermiform adult female and sedentary female stages. Such data could serve as a platform for designing RNAi experiments to silence putative parasitism genes in vermiform adult female and sedentary female stages, thus resulting in a lower rate of infection.

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